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Response of mesangial cells to low-density lipoprotein and angiotensin II in diabetic (OLETF) rats

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Response of mesangial cells to low-density lipoprotein and angiotensin II in diabetic (OLETF) rats.

Backgound. Progression of diabetic nephropathy is closely associated with morphological changes in glomeruli, such as thickening of the glomerular basement membrane, mesangial expansion, and glomerulosclerosis. To elucidate early glomerular events, we compared the mitogenic activity and extracellular matrix production in mesangial cells (MC) isolated from diabetic rats prior to the manifestation of nephropathy and those showing overt nephropathy. This study may help to clarify the mechanisms underlying diabetic nephropathy and provide clues about early therapeutic interventions for preventing or slowing this process.

Methods. Otsuka Long-Evans Tokushima Fatty (OLETF) rats, a chronic model for human type 2 diabetes mellitus, and age-matched control (LETO) rats were used. Glomerular cell numbers, expression of immediate early genes (c-Fos and c-Myc) and proliferating cell nuclear antigen (PCNA), and low-density lipoprotein (LDL) deposition were determined in renal tissue sections from rats aged 15 to 75 weeks. Mesangial cells (MCs) from OLETF rats at two different stages of the disease, that is, young (12- to 14-week-old) OLETF rats (y-OLETF) prior to the manifestation of nephropathy and old (48- to 50-week-old) OLETF rats (o-OLETF) showing nephropathy, were isolated and cultured. After stimulation with native (n-) or oxidized (ox-) LDL or angiotensin II (Ang II), DNA synthesis and extracellular matrix (ECM) production were examined. Cellular expression of LDL/scavenger receptors was analyzed using fluorescence-labeled LDL and binding to ^{125}I-labeled-LDL.

Results. The number of cells per glomerular cross section was significantly higher in OLETF rats than in LETO rats between 25 and 65 weeks of age. In OLETF glomeruli, c-Fos, c-Myc, and PCNA were transiently expressed in the early phase. Glomerular LDL deposition increased with the age of OLETF rats. Addition of a low dose of n-LDL (10 μg/mL) to the culture medium significantly stimulated DNA synthesis of y-OLETF MCs, as compared with o-OLETF MCs and LETO MCs ($P < 0.05$). A high dose of n-LDL (100 μg/mL) caused cytotoxic effects in all cells. Exposure to ox-LDL minimally affected DNA synthesis of OLETF or LETO MCs. LDL receptors and scavenger receptors were predominant in y-OLETF and o-OLETF, respectively. After stimulation with n-LDL and ox-LDL, expression of type I and type III collagen, along with transforming growth factor-β (TGF-β), was higher in o-OLETF MCs that in y-OLETF MCs or LETO MCs. Exposure to Ang II markedly induced DNA synthesis and ECM mRNA expression in y-OLETF MCs and o-OLETF MCs, respectively.

Conclusions. These findings indicate that the cell proliferation process precedes the evolution of diabetic glomerulopathy. The responses of OLETF MCs to n-LDL/ox-LDL and Ang II differed depending on the stage of diabetes. In the early phase, MCs were prone to proliferate, whereas in the late stage, MCs, which expressed higher levels of TGF-β, tended to synthesize ECM. A functional switch in MCs may contribute to the development of glomerulosclerosis in diabetic nephropathy.

Thirty to 50% of patients with juvenile- or mature-onset diabetes mellitus develop clinically evident diabetic nephropathy 10 to 20 years from the onset of diabetes mellitus [1]. Thus, it is important to elucidate the mechanisms involved in the progression of renal complications of this disease. Diabetic nephropathy is characterized by glomerular hypertrophy, extracellular matrix (ECM) accumulation in the mesangium, and thickening of glomerular capillary walls, leading to glomerulosclerosis and loss of renal function [2, 3].

Rats administered streptozotocin or alloxan and genetically selected animals fed a high-sucrose diet have been employed as the models for type 2 diabetes mellitus [4]. In this study, we used the Otsuka Long-Evans Tokushima Fatty (OLETF) rat, which was developed from a strain of Long-Evans rat by selective breeding and is now recognized as an adequate model for human type 2 diabetes mellitus [5, 6]. The characteristic features of OLETF rats are [5, 6] (*1*) a chronic course of diabetes; (*2*) hyperlipidemia; (*3*) mild obesity; (*4*) male inheritance; (*5*) hyperplastic foci of pancreatic islets; and (*6*) renal complications. In OLETF rats, plasma glucose levels are high at 8 weeks of age and then significantly exceed that of the genetic controls, the Long-Evans Tokushima Otsuka (LETO) rats, after 18 weeks of age. Hypercholesterolemia and hypertriglycemia appear at 8 weeks of

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age, but deteriorate with age. OLETF rats past the age of 30 weeks show significant proteinuria compared with LETO rats, and the proteinuria progressively increases with time [5, 6]. Pathological changes of the kidney in OLETF rats past the age of 40 weeks include ECM accumulation in the mesangium, thickening of the glomerular basement membrane, and glomerulosclerosis, changes resembling human diabetic glomerulopathy [5, 6]. Thus, this model is appropriate for observing the long-term disease course.

Several growth factors or metabolic products, including transforming growth factor-β (TGF-β), insulin-like growth factor-I, platelet-derived growth factor, prostaglandins, angiotensin II (Ang II), low-density lipoprotein (LDL) and advanced glycation end products, have been shown to be contributing factors involved in the progression of diabetic glomerulopathy [7–9]. These factors can promote mesangial cell (MC) proliferation, glomerular hypertrophy, and ECM overproduction, or alter the glomerular hemodynamics.

The present study was performed to elucidate the early events in the evolution of diabetic glomerulopathy. MCs from OLETF rats at two different stages of the disease, that is, MCs from young rats prior to development of nephropathy at 12 to 14 weeks old (y-OLETF MC) and MC from old rats showing overt nephropathy at 48 to 50 weeks old (o-OLETF MC), were compared with respects to their response to native (n)-LDL/oxidized (ox)-LDL and Ang II.

METHODS

Animals and reagents

All procedures were in accordance with institutional guidelines for animal research. Male OLETF rats and control male LETO rats were supplied by Otsuka Pharmaceutical Co., Ltd. (Tokushima, Japan). They were fed standard laboratory chow and given tap water ad libitum. Human n-LDL and ox-LDL were purchased from Biogenesis (Sandown, NH, USA). n-LDL labeled with 1,1′-dioactadecyl-3,3,3′-tetramethylindocarbocyanine perchlorate (Dil-n-LDL) and Dil-acetylated-LDL were from Biomedical Technologies Inc. (Stoughton, MA, USA) and Harbor Bio-Products (Norwood, MA, USA), respectively. [^{125}I]-sodium salt, [^{3}H]-thymidine and [^{32}P]-dCTP were obtained from Amersham Japan (Tokyo, Japan). Rabbit antibody to LDL (reactive with n-LDL, but not with ox-LDL) was purchased from Alpha Biomedical Laboratories (Bellevue, Washington, USA). Mouse antibody to proliferating cell nuclear antigen (PCNA) was from Chemicon (Temecula, CA, USA). Rabbit antibodies to c-Fos and c-Myc were from Oncogene Science (Cambridge, MA, USA). Fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit IgG and FITC-labeled goat anti-mouse IgG were purchased from Cappel (Durham, NC, USA). Rabbit antibodies to rat collagen types I and III were from Chemicon. Fetal calf serum (FCS) was purchased from HyClone (Logan, UT, USA). The following reagents were purchased from Sigma (St Louis, MO, USA): Ang II, trypsin, collagenase, RPMI 1640, Dulbecco's modified Eagle's medium (DMEM), ampicillin-streptomycin solution, insulin, Nu-serum and fatty acid-free bovine serum albumin (BSA). Salmon sperm DNA was obtained from Stratagene (Heidelberg, Germany). 50 × Denhardt's solution was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Glomerular isolation and MC culture

Mesangial cells were separated from glomeruli of male OLETF rats according to the procedure described by Harper et al [10] as described in our earlier publications [11, 12]. Animals at two different stages of the disease were selected: young rats (y-OLETF) prior to development of nephropathy at 12 to 14 weeks of age and weighing 400 to 500 g, and old rats (o-OLETF) showing developed nephropathy at 48 to 50 weeks of age that weighed 600 to 700 g. Glomeruli were harvested by sieving the renal cortices, which were then digested with 0.25% trypsin, followed by incubation with 0.05% collagenase for 10 to 15 minutes each at 37°C in culture chambers. As the primary culture, the digested glomeruli were incubated at 37°C in RPMI 1640 supplemented with 20% FCS, 100 U/mL ampicillin, 100 μg/mL streptomycin and 0.6 U/mL insulin. After two to three weeks, MCs appeared and obliterated the epithelial cells. Cultured cells were detached with trypsin/ethylenediaminetetraacetic acid (EDTA), and glomeruli were removed by filtration. Mesangial cells were characterized by their typical stellate morphology on light microscopy, positive staining for smooth muscle-specific actin, and negative staining for factor VIII antigen and for cytokeratin, as described previously [11, 12]. Mesangial cells from control LETO rats, 12 to 14 weeks old and weighing 400 to 450 g (y-LETO), or 48 to 50 weeks old and weighing 500 to 550 g (o-LETO), also were cultured. LETO rats never became diabetic. MCs were expanded and passaged every seven days, and the cells between the fifth and eighth passages were utilized in this study. The cells maintained the characteristics of MCs described above. Confluent cells were made quiescent by incubation in serum-free medium for 24 to 48 hours before the addition of test agents.

Assay of DNA synthesis

Rat MCs, cultured in 24-well plastic plates until confluence and starved in FCS-free RPMI 1640 for 48 hours, were incubated with n-LDL/ox-LDL, Ang II or FCS (for baseline line culture) for the indicated times. [^{3}H]-thymidine was added to the cultures at a final concentration of 1 μCi/mL and the cultures were incubated for an additional four hours. The cells were rinsed three times

with ice-cold phosphate-buffered saline (PBS) followed by incubation with 10% trichloroacetic acid for 30 minutes on ice. After rinsing with 100% ethanol, acid-precipitable materials were dissolved in 0.25 N NaOH/0.1% sodium dodecyl sulfate (SDS) and counted by scintillation spectrometry to determine the incorporation of [^{3}H]-thymidine into the acid-insoluble cellular DNA fraction [13].

RNA isolation and Northern blot analysis

Total RNA was extracted from cultured MC using Isogen solution (Nippon Gene, Tokyo, Japan). Aliquots of 15 μg of RNA were electrophoresed in each lane, transferred onto S & S Nytran nylon membranes (Schleicher & Schuell, Keene, NH, USA), and fixed by exposure to UV light (Funakoshi Ltd, Tokyo, Japan). The membranes were prehybridized in a solution consisting of 50% formamide, 5 × standard sodium citrate (SSC), 0.5% (wt/vol) SDS, 5 × Denhardt's solution and 100 mg/mL sonicated salmon sperm DNA at 42°C for at least four hours. Blots were then hybridized with 0.5 to 1.0 × 10^7 cpm/mL of ^{32}P-labeled cDNA probes at 42°C for 12 to 18 hours. The cDNA probes used were the 1.3-kb PstI-BamHI fragment of rat type I collagen, 2.23-kb EcoRI-BamHI fragment of rat type III collagen, rat transforming growth factor-β (a gift from Dr. Enyu Imai, The First Department of Internal Medicine, Osaka University Graduate School of Medicine, Osaka, Japan) [14], and 2.2-kb EcoRI fragment of matrix metalloproteinase (MMP)-2 (a gift from Dr. R.C. Harris, Department of Medicine, Vanderbilt University School of Medicine, Nashville, TN, USA). Probes were labeled with ^{32}P-dCTP (>10^8 cpm/μg) by the random priming method using the Megaprime DNA Labeling System (Amersham, Buckinghamshire, UK). After washing twice with 2 × SSC for 15 minutes at room temperature and with 1 × SSC containing 0.1% SDS for 15 minutes at 65°C, and finally with 0.2% SSC containing 0.1% SDS for an additional 15 minutes, the membranes were exposed to Amersham Hyper-ECl film at −70°C with intensifying screens. The blots were stripped and reprobed with a human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (Clontech, Heidelberg, Germany) under the same conditions described above. Expression levels of mRNA were quantified by densitometry and normalized relative to GAPDH expression. Data were presented as means ± SE of the ratios of optical density (OD) areas for mRNAs, divided by OD areas for GAPDH mRNA.

[^{125}I]-LDL binding assay

[^{125}I]-Low-density lipoprotein was prepared using a commercial kit (Iodo-Gen; Pierce, Rockford, IL, USA). Free [^{125}I] was removed by gel filtration. The final radioactivities ranged from 0.5 × 10^6 to 1 × 10^7 CPM/μg LDL. The LDL binding assay was carried out according to the method described by Goldstein, Basu and Brown [15] with slight modifications [16]. After reaching confluence, MCs were incubated in medium containing lipoprotein-deficient serum consisting of DMEM supplemented with 1% FCS, 8% Nu-serum and 1% ampicillin-streptomycin solution for 36 hours before the experiment. The medium was then removed and replaced with 1 mL of medium consisting of Dulbecco's PBS with 100 mg% of glucose, 2 mg/mL fatty acid-free BSA and [^{125}I]-labeled n-LDL or ox-LDL (1 × 10^6 CPM/mL). After incubation at 4°C for the times indicated, the medium was removed, and the MCs were washed three times with ice-cold PBS containing 1% BSA. Additional solution was added to the culture plates, the plates were incubated for five minutes, and the solution was removed. Finally, cells were washed with PBS without BSA. Then, the cells were osmolyzed in 1.0 N NaCl and radioactivity was counted with a gamma counter.

Incorporation of LDL into MCs

Incorporation of LDL into MCs was examined using a Dil-labeled fluorescent probe as described previously [16]. Subconfluent MCs were incubated with Dulbecco's PBS with 100 mg% glucose and either 10 μg/mL of Dil-n-LDL or Dil-acetylated-LDL at 37°C for two hours. The cells were washed vigorously with PBS and examined by immunofluorescence microscopy.

Morphological and immunohistological studies

OLETF and LETO rats were sacrificed and studied at 15, 25, 35, 45, 55, 65, and 75 weeks. Each group consisted of five rats. The number of intraglomerular cells per glomerular cross-section was assessed by counting the total cell nuclei under a microscope using PAS-stained sections containing at least 20 equatorially cut glomeruli from each animal, as described previously [17–19]. Indirect immunofluorescent staining was performed as described previously [20, 21]. In brief, sections were reacted with the primary antibody followed by incubation with the appropriate secondary antibodies labeled with FITC. As a control, kidney sections were incubated with non-immune rabbit serum or unrelated mouse IgG antibody, followed by appropriate FITC-labeled secondary antibodies or secondary antibody alone. These controls gave entirely negative results. The sections containing at least 20 glomeruli ($N = 5$) were viewed under an immunofluorescent or a phase-contrast immunofluorescent microscope (Nikon Eclipse E600, Tokyo, Japan) and the glomerular cells immunoreactive for PCNA, c-Fos, or c-Myc were counted, as described previously [18, 22]. The data were expressed as the number of positive cells per glomerular cross-section.

Statistics

Data are presented as mean ± SE. Statistical comparisons were performed using the Mann-Whitney U test with the software program StatView 4.5. $P < 0.05$ was considered statistically significant.

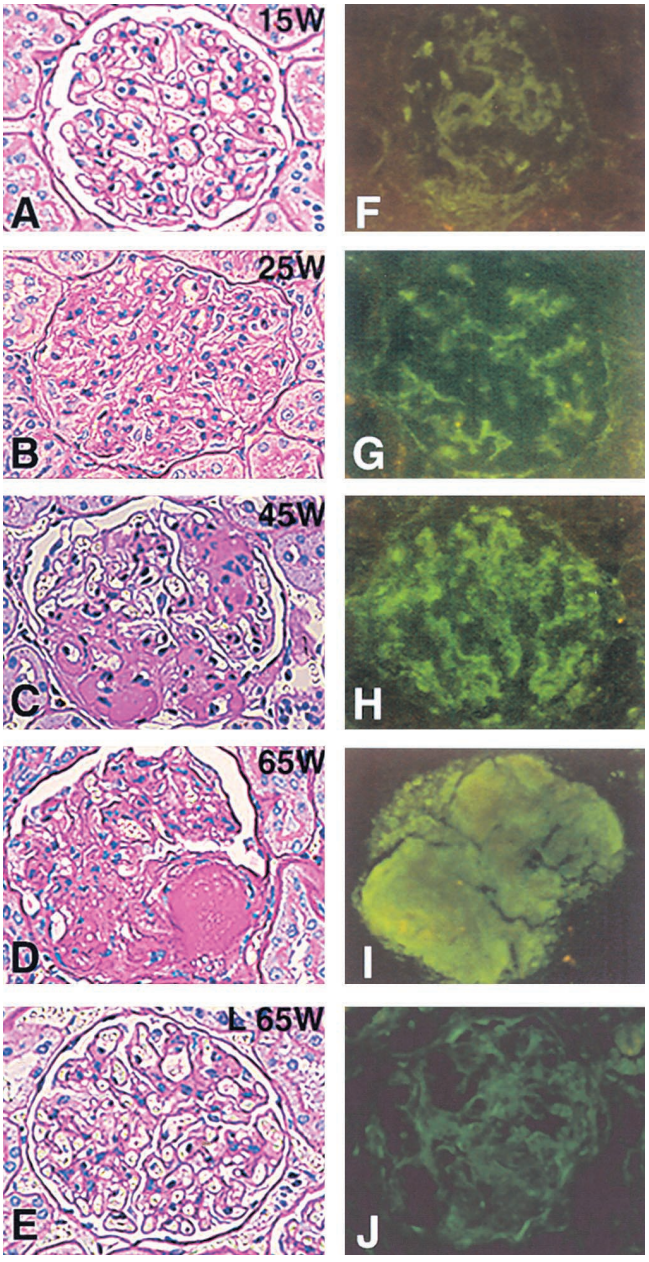

Fig. 1. Microscopic findings (*A–E*) and low-density lipoprotein (LDL) deposition (*F–J*) in glomeruli of Otsuka Long-Evans Tokushima Fatty (OLETF) and Long-Evans Tokushima Otsuka (LETO) rats. Glomerular changes were minimal in 15-week-old OLETF rats (A). An increase of mesangial cells was seen in 25-week-old OLETF rats (B). ECM accumulation was evident in 45-week-old OLETF rats (C) and "nodular lesions" were present in 65-week-old OLETF rats (D), while only minor glomerular changes were seen in 65-week-old LETO rats (E). Glomerular LDL deposition was seen in 15- (F) and 25-week-old (G) OLETF rats. LDL deposition was intense in glomeruli of 45- (*H*) and 65-week-old (*I*) OLETF rats, whereas it was weak in glomeruli of 65-week-old LETO rats (*J*). Original magnifications were ×400.

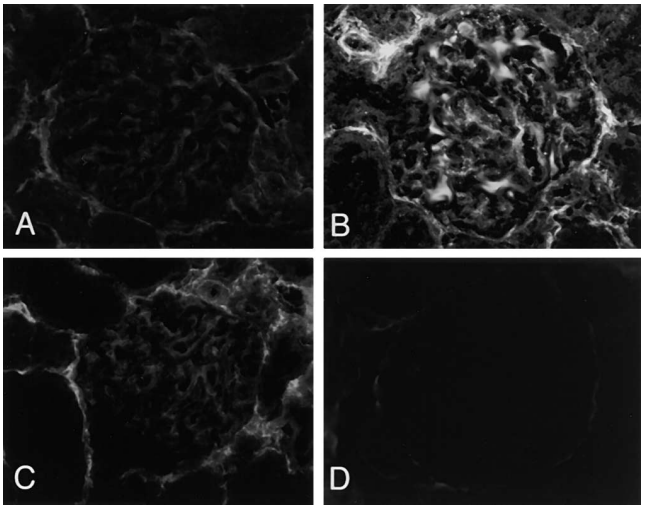

Fig. 2. Immunofluorescent staining for type III collagen in OLETF and LETO rat glomeruli. The accumulation of type III collagen was evident in the mesangial areas in 45-week-old OLETF rats (*B*), but much less evident in 15-week-old OLETF (*A*) and 45-week-old LETO (*C*) rats. A negative control section of a 45-week-old OLETF rat, stained with non-immune rabbit serum, followed by FITC-labeled secondary antibodies, is shown in (*D*). Original magnifications were ×400.

RESULTS

Morphology of glomeruli of OLETF rats

Representative light micrographs of glomeruli of OLETF and control LETO rats of various ages are shown in Figure 1 A–E. In 15-week-old OLETF rats, there were no obvious alterations in glomeruli. At 25 weeks of age, MC proliferation was observed. After 45 weeks, mesangial expansion accompanied by the accumulation of ECM and thickening of glomerular capillary walls occurred. At 65 weeks, fully developed diabetic glomerulopathy accompanied by nodular sclerosis was seen. In LETO rats, there were no obvious findings except minor alterations due to aging in glomeruli even at 65 weeks.

ECM and LDL deposition in glomeruli of OLETF rats

Glomerular deposition of LDL was weak in 15-week-old OLETF rats, and it progressively increased from 25 to 65 weeks of age (Fig. 1 F–I). In LETO rat glomeruli, LDL was negative from 15 to 45 weeks of age and was weakly deposited after 65 weeks of age (Fig. 1J). Type I and type III collagens were accumulated in the glomeruli in 45- to 65-week-old OLETF rats, but were not as extensively accumulated in the age-matched controls (Fig. 2).

Glomerular cell number and expression of PCNA, c-Fos and c-Myc in OLETF rats

Total glomerular cellularity and the glomerular cells expressing PCNA, c-Fos and c-Myc were assessed using kidney sections from OLETF and LETO rats (Fig. 3). Glomerular cell numbers were significantly higher in OLETF rats from 25 to 65 weeks of age than in age-matched LETO rats. Glomerular cells expressing PCNA

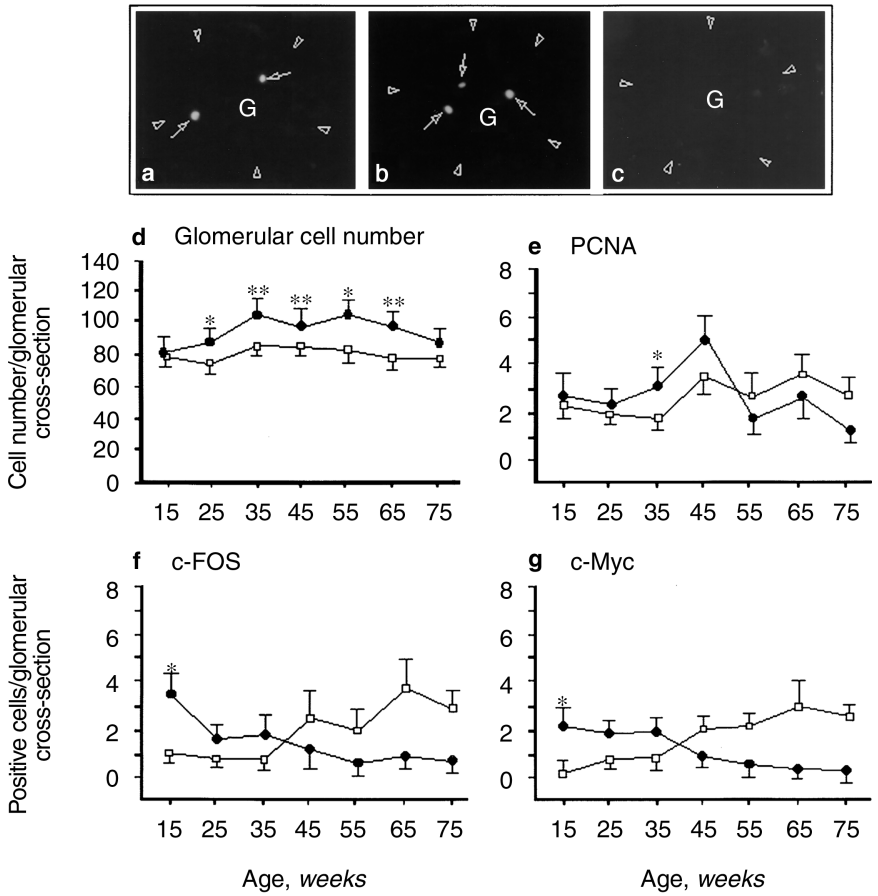

Fig. 3. Total glomerular cellularity and the glomerular cells expressing PCNA, c-Fos and c-Myc in OLETF rat kidneys. Immunofluorescent staining for PCNA (*a*) and c-Myc (*b*) in glomeruli of 35-week-old and 15-week-old OLETF rats, respectively. Note the diffuse nuclear staining (arrows) within the glomeruli. A negative control section, stained with nonimmune rabbit serum, followed by FITC-labeled secondary antibodies, of 35-week-old OLETF rat is shown in (*c*). Arrowheads in panels a to c denote the Bowman's capsule; G is glomerulus. Original magnifications were ×400. Cell number (*d*) and expression of PCNA (*e*), c-Fos (*f*), and c-Myc (*g*) in glomeruli of OLETF (■) and LETO (□) rats. Glomerular cell numbers were significantly higher in OLETF than in LETO rats between 25 and 65 weeks of age (d). PCNA expression (e) in glomeruli was significantly increased in 35-week-old OLETF rats compared with that in LETO rats. The number of glomerular cells expressing c-Fos (f) and c-Myc (g) was significantly higher in 15-week-old of OLETF rats than in LETO rats. Total glomerular cells and glomerular cells positive for PCNA, c-Fos, and c-Myc were counted in at least 20 glomeruli in each section ($N = 5$ per group). Data are presented as mean ± SE. $^{*}P < 0.05$, $^{**}P < 0.01$.

were significantly increased in OLETF compared to LETO rats at 35 week of age. Immunostaining revealed that the number of c-Fos- or c-Myc-positive glomerular cells was significantly higher in OLETF rats than in LETO rats at 15 weeks of age.

Effects of LDL on DNA synthesis in cultured MCs

In cultures of MCs in 10% FCS, the growth rate of MCs from y-OLETF rats was similar to that of MCs from o-OLETF, y-OLETF and o-LETO rats for up to 48 hours. From 72 to 120 hours, the growth of MCs from y-OLETF rats was faster, as shown by an approximately 1.6-fold increase in cell numbers compared with y- and o-LETO MCs, and an approximately 1.3-fold increase compared with o-OLETF MCs.

Mesangial cells, cultured until confluent and starved in FCS-free medium for 48 hours, were incubated with 10 μg/mL, 50 μg/mL, or 100 μg/mL of n-LDL or ox-LDL (Fig. 4). DNA synthesis evaluated by [^{3}H]-thymidine incorporation was compared between LDL-stimulated cells and unstimulated controls (=100%). The results are expressed as the percent of the control DNA synthesis. After exposure of MCs to 10 μg/mL of n-LDL, DNA synthesis in y-OLETF MCs increased to approximately 300% of control level. DNA synthesis in o-OLETF, y-LETO and o-LETO MCs increased to 180 to 200% of the control level; the difference between y-OLETF MCs and o-OLETF or LETO MCs was significant ($P < 0.05$). DNA synthesis in MCs from OLETF and LETO rats was weakly stimulated by 50 μg/mL of n-LDL. The addition of 100 μg/mL of n-LDL suppressed DNA synthesis in OLETF and LETO MCs, while the addition of 10 μg/mL, 50 μg/mL, or 100 μg/mL of ox-LDL minimally affected DNA synthesis in OLETF and LETO MCs.

Analysis of LDL receptors/scavenger receptors in MC

Incorporation of fluorescence-labeled n-LDL/acetylated LDL and binding of ^{125}I-labeled n-LDL/ox-LDL to MCs were studied. After a two-hour incubation, fluorescence of Dil-n-LDL was strong in y-OLETF MCs, but weak in o-OLETF or LETO MCs (Fig. 5A). Binding of ^{125}I-labeled n-LDL to MCs reached equilibrium after one to two hours of incubation. The maximum binding to y-OLETF MCs was approximately twofold greater than that to LETO MCs and fourfold greater than that to o-OLETF MCs (Fig. 5B). Addition of excess amounts of unlabeled n-LDL (100 μg/mL) displaced approxi-

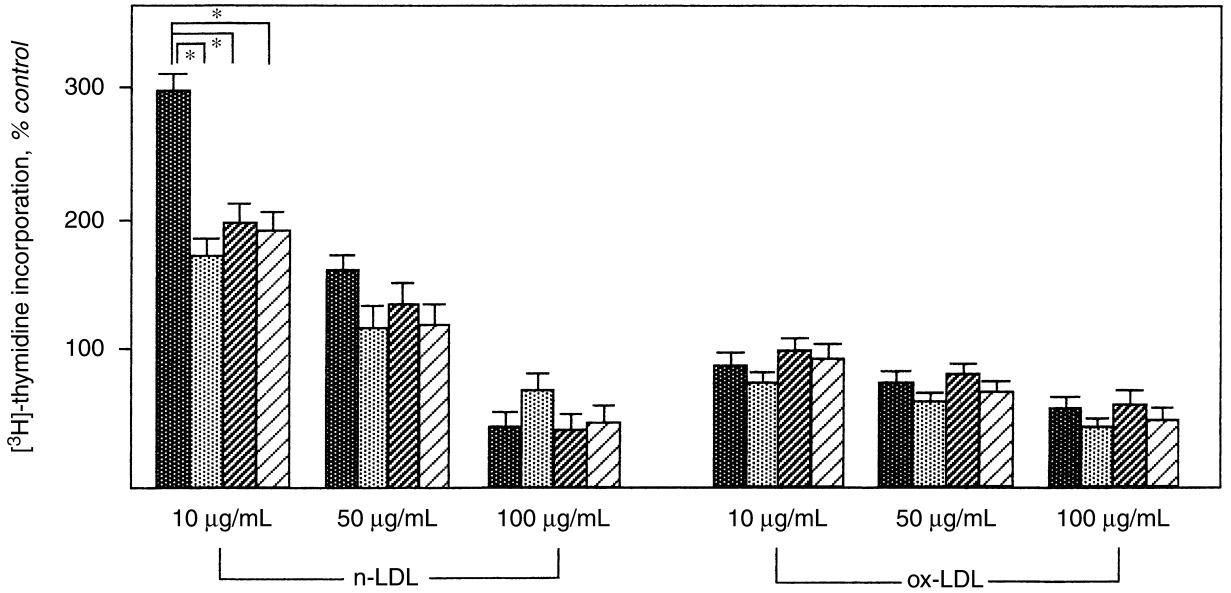

Fig. 4. DNA synthesis in mesangial cells (MCs) exposed to native (n)-low-density lipoprotein (LDL)/oxidized (ox)-LDL. MCs were incubated with 10 μg/mL, 50 μg/mL, or 100 μg/mL of n-LDL or ox-LDL for 4 hours. The unstimulated basal level in young (y)-OLETF MCs (■), old (o)-OLETF (▩), y-LETO (▨), and o-LETO (▨) was 2.3×10^4 cpm, 0.8×10^4 cpm, 1.0×10^4 cpm, and 0.9×10^4 cpm, respectively. After stimulation with 10 μg/mL of LDL, [^{3}H]-thymidine incorporation in y-OLETF, o-OLETF, y-LETO, and o-LETO was 7.0×10^4 (mean) cpm, 1.5×10^4 cpm, 2.1×10^4 cpm, and 1.9×10^4 cpm, respectively. y-OLETF MCs showed an approximately 300% increase from the unstimulated basal level, compared with o-OLETF MCs (~180% increase) or control LETO MC (~200% increase). DNA synthesis in MCs from OLETF and LETO rats was weakly stimulated by 50 μg/mL of n-LDL, while 100 μg/mL of n-LDL suppressed DNA synthesis in MCs from OLETF and LETO rats. Exposure to 10 μg/mL, 50 μg/mL or 100 μg/mL of ox-LDL did not markedly affect DNA synthesis in OLETF and LETO MCs. Symbols and bars represent means ± SE of 3 experiments, each carried out in quadruplicate; $*P < 0.05$.

mately 40 to 50% of the ^{125}I-labeled LDL in a time-dependent manner.

Similarly, incorporation of fluorescence-labeled acetylated-LDL and binding of ^{125}I-labeled ox-LDL to MCs were studied. Uptake of Dil-acetylated-LDL was high in o-OLETF MCs (Fig. 6A). The level of ^{125}I-ox-LDL binding was elevated in o-OLETF MCs (1.5-fold and 3 to ~4-fold higher in o-OLETF MCs than in y-OLETF and LETO MCs, respectively).

ECM production by MCs after stimulation with n-LDL/ox-LDL

Type I and type III collagen synthesis in MCs after stimulation with 50 μg/mL of n-LDL or ox-LDL was analyzed by Northern blotting (Fig. 7). In o-OLETF MCs, type I collagen mRNA expression was up-regulated after addition of n-LDL or ox-LDL. y-OLETF MCs and LETO MCs showed only a slight increase in type I collagen mRNA levels. Similar results were observed for type III collagen mRNA expression in these cells (data not shown).

Induction of TGF-β mRNA and MMP-2 mRNA expression by n-LDL/ox-LDL

Northern blot analyses revealed that TGF-β mRNA expression in MCs from OLETF and LETO rats was minimal in untreated cultures in FCS, but was markedly elevated in o-OLETF MCs compared with y-OLETF or LETO MCs after stimulation with 50 μg/mL n-LDL or ox-LDL (Fig. 8). Induction of MMP-2 mRNA by n-LDL/ox-LDL was similar in y-OLEFT, o-OLETF, and LETO MCs (data not shown).

Effects of Ang II on DNA synthesis and ECM production in MC

Exposure to Ang II (10^{-7} mol/L) caused a significantly greater elevation of DNA synthesis in y-OLETF MCs (300% of control level in y-OLETF vs. 120 to 160% of control level in o-OLETF, y-LETO and o-LETO, $P < 0.05$; Fig. 9). Northern blot analyses for type I and III collagen mRNA revealed that ECM production was high in unstimulated o-OLETF MCs and was further increased after incubation with Ang II (Fig. 10A), while there was no marked increase in y-OLETO or LETO. After stimulation of MCs with Ang II, TGF-β mRNA was also more markedly up-regulated in o-OLETF MCs than in MCs from y-OLETF or LETO rats (Fig. 10B).

DISCUSSION

Using OLETF rats, which exhibit chronic-course diabetes resembling human type 2 diabetes mellitus, we found a significant increase in glomerular cell number in OLETF compared to LETO rats between 25 and 75 weeks of

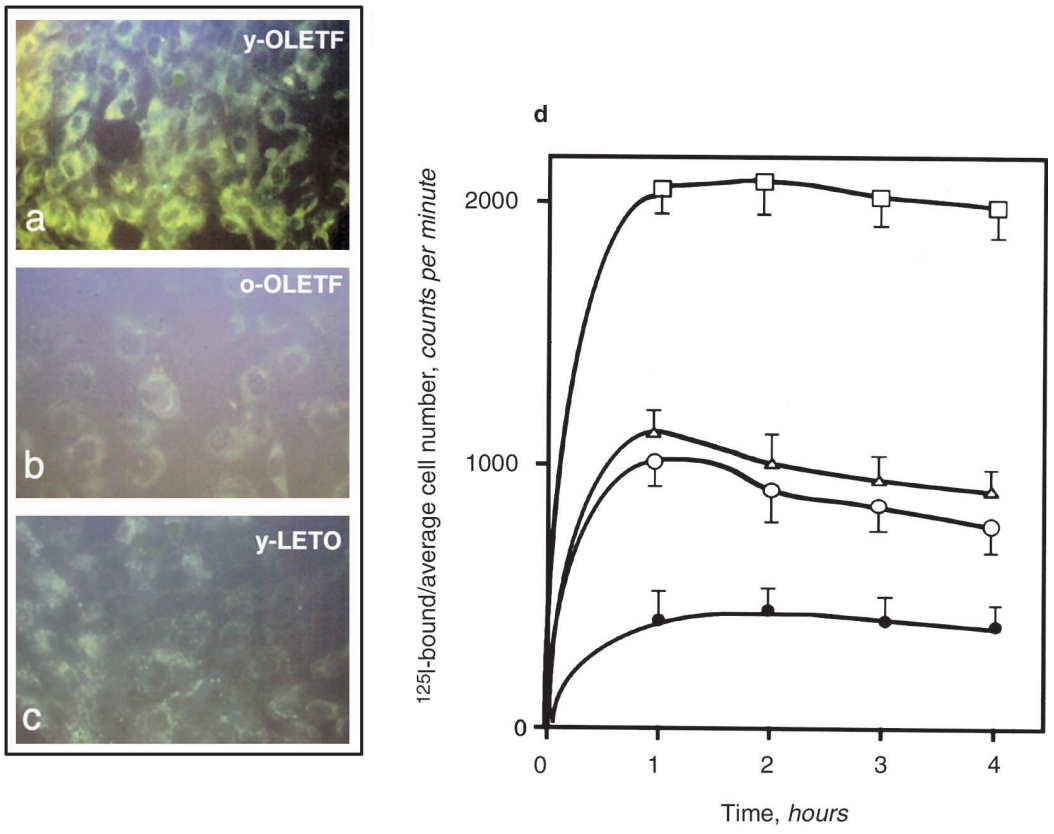

Fig. 5. Uptake of fluorescence-labeled n-LDL into cultured MCs and binding of ^{125}I-labeled n-LDL to cultured MCs. Dil-n-LDL-uptake (*a*) and binding to ^{125}I-labeled n-LDL (*b*) were higher in y-OLETF MCs (*c*) than in o-OLETF or LETO MCs. Symbols and bars and bars represent means ± SE of 3 experiments, each carried out in quadruplicate. Symbols in panel *d* are: (□) y-OLETF; (△) y-LETO; (○) o-LETO; (●) o-OLETF.

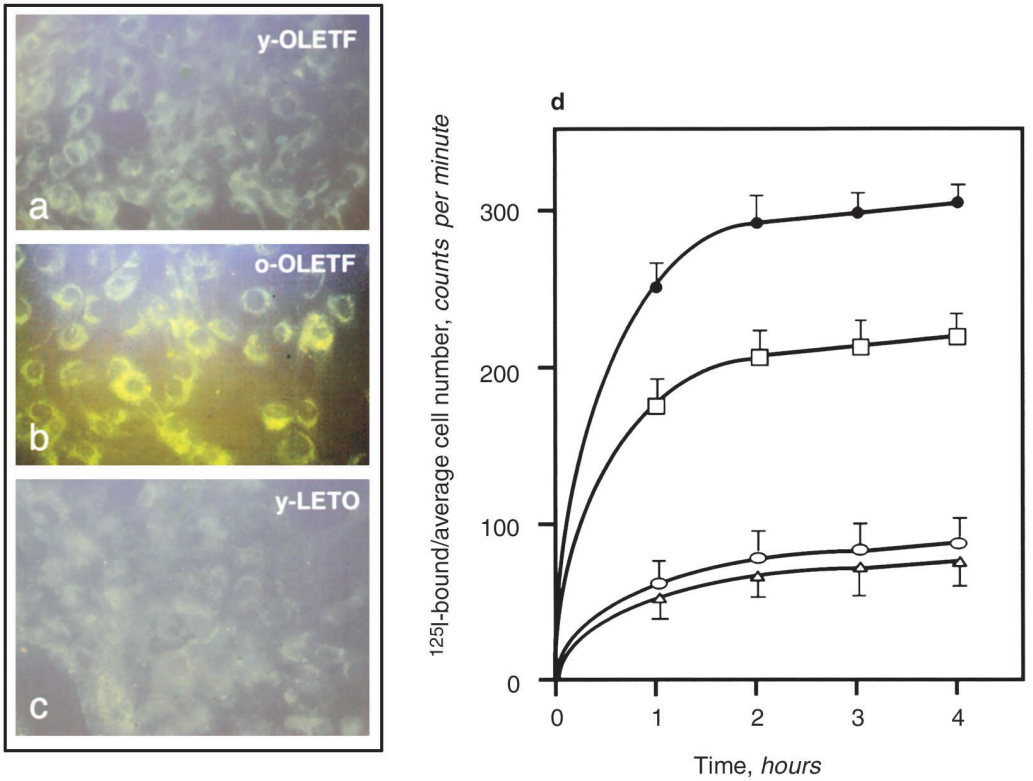

Fig. 6. Uptake of fluorescence-labeled modified LDL into cultured MCs and binding of ^{125}I-labeled ox-LDL to cultured MCs. Dil-acetylated-LDL uptake (*a*) and ^{125}I-labeled ox-LDL binding (*b*) were elevated in o-OLETF MCs compared with y-OLETF or LETO MCs. Symbols and bars and bars represent means ± SE of 3 experiments, each carried out in quadruplicate. Symbols in panel *d* are: (●) o-OLETF; (□) y-OLETF; (○) o-LETO; (△) y-LETO.

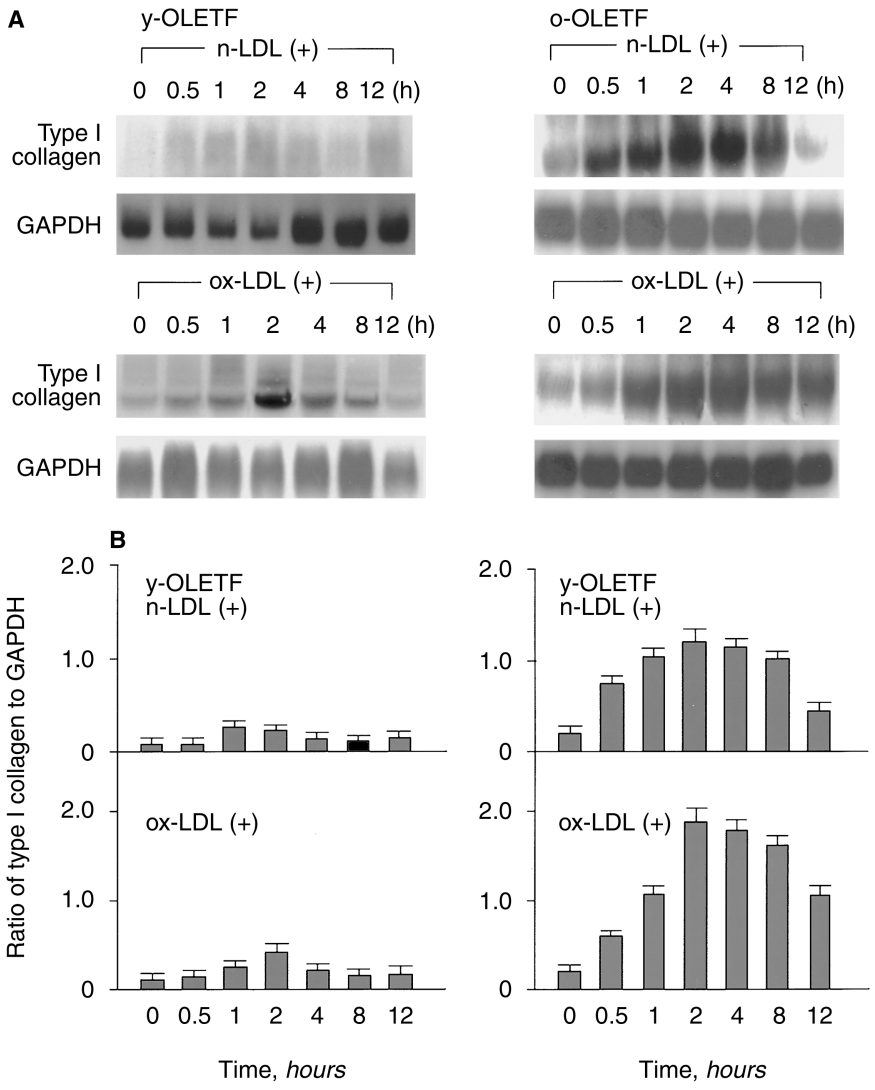

Fig. 7. Type I collagen mRNA expression in MCs after exposure to n-LDL or ox-LDL. (*A*) Northern blots of representative experiments and (*B*) the ratio of optical density (OD) areas for type I collagen mRNA divided by OD areas for DAPDH mRNA. Symbols and bars represent means ± SE of 3 experiments, each carried out in quadruplicate. After exposure to 50 μg/mL of n-LDL or ox-LDL, type I collagen mRNA expression in o-OLETF MCs was up-regulated and peaked within 2 to 8 hours of incubation (right panel). Type I collagen mRNA expression was only slightly increased in y-OLETF MCs (left panel) and OLETO MCs (data not shown) after stimulation with n-LDL or ox-LDL.

age. Glomerular cells in OLETF rats in the early stage showed transient up-regulation of PCNA as well as of the immediate early gene products c-Fos and c-Myc. Previous studies investigating glomerular cell proliferation in the early phase of diabetes reported conflicting results. Earlier morphological observations performed using kidneys from patients with type I diabetes mellitus [23, 24] or streptozotocin-induced diabetic rats [25] revealed glomerular hypertrophy and basement membrane thickening as the initial structural alterations. Subsequent detailed studies using autoradiography indicated that glomerular hypercellularity occurs concomitantly with glomerular enlargement in the early phase of streptozotocin-induced diabetes in rats [26]. Mahadevan et al reported a significant increase in the number of MC in rats 21 days after induction of streptozotocin-induced diabetes [27]. PCNA, a nuclear protein that is maximally activated during the S-phase of the cell cycle [28], and immediate early genes (c-fos, c-myc, and Egr-1), transcriptional factors that are expressed in the G_1 phase of the cell cycle [29], have been studied in diabetic models [27, 30, 31] and MCs cultured in high-glucose media [32]. Increased expression of PCNA has been reported in glomerular cells from rats with streptozotocin-induced diabetes [19, 30]. Young et al observed early and self-limited MC proliferation in this model, and showed that staining for PCNA was evident by day 1, and peaked at day 3 [19]. PCNA as well as immediate early genes (c-myc and Egr-1) were induced in MCs cultured in high glucose conditions [32]. Transient increases in c-fos and c-jun were reported after the onset of hyperglycemia in diabetic rats [31]. Thus, our data, together with those of others [19, 27, 30–32], indicate that proliferation of glomerular cells occurs in the diabetic milieu preceding the development of nephropathy.

Abnormal lipid metabolism is a frequent complication

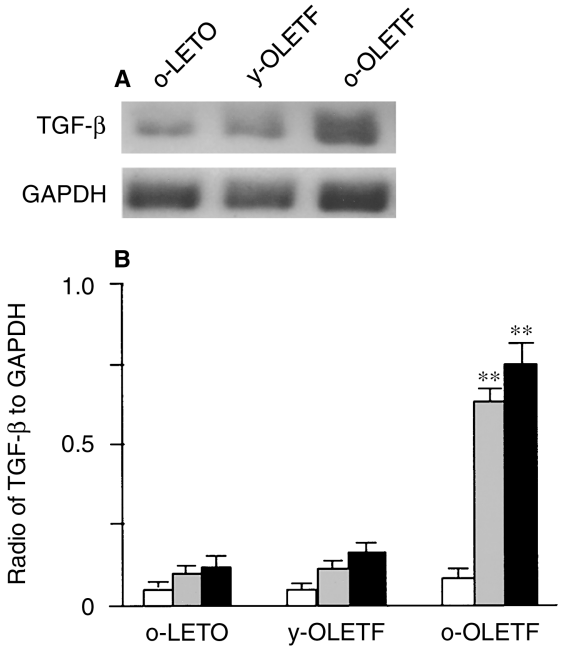

Fig. 8. Transforming growth factor-β (TGF-β) mRNA expression in MCs treated with n-LDL/ox-LDL. (*A*) Northern blot analysis showed that, after 18 hours of incubation with 50 μg/mL of ox-LDL, TGF-β mRNA expression was higher in o-OLETF MCs than in y-OLETF or LETO MCs. (*B*) The ratio of TGF-β mRNA signal to GAPDH mRNA signal in OLETF and LETO MCs with stimulation by 50 μg/mL of n-LDL (▩) or ox-LDL (■) or or without stimulation of LDL (□). n-LDL and ox-LDL significantly increased TGF-β mRNA expression in o-OLETF, compared with y-OLETF ($P < 0.01$) and o-LETO ($P < 0.01$). Symbols and bars represent means ± SE of 3 experiments, each carried out in quadruplicate; $^{**}P < 0.01$.

in various types of immune-mediated or nonimmune-mediated glomerular diseases [33, 34]. Previous studies in humans and animals have suggested that altered lipid metabolism causes glomerular injury and promotes deterioration of glomerular function [35, 36]. Hypercholesterolemia and hypertriglycemia, commonly observed during the course of human diabetes mellitus [37], are also found in OLETF rats [5, 6]. Quantitative abnormalities of the major cholesterol carrier LDL as well as qualitative alterations of LDL such as enhanced glycation and oxidation have been implicated in diabetic microvascular complications [38, 39]. Glomerular cells, including MCs and epithelial cells, possess LDL receptors, and thus are affected by abnormal lipid metabolism [16, 40]. In addition, MCs can express scavenger receptors [21, 41]. As shown for the development of atherosclerosis [42], glomerular cells can be damaged by the uptake of n-LDL or ox-LDL via their specific receptors.

We studied the glomerular LDL deposition and the response of OLETF MCs to n-LDL or ox-LDL. In this model, plasma levels of cholesterol and triglyceride increased from 8 weeks of age [5, 6]. We found that glomerular LDL deposition progressively increased with age, whereas the LDL receptor in o-OLETF rats appeared to be down-regulated. These observations suggest that, in addition to LDL binding to its specific receptor, other mechanisms, such as affinity of LDL to glycosaminoglycans in glomerular matrix components [43] or phagocytosis of LDL by mesangial cells [44], are involved in glomerular LDL deposition.

Young-OLETF MCs, expressing high levels of LDL receptors, markedly proliferated after exposure to low concentrations (10 μg/mL) of n-LDL. In contrast, proliferative response to this dose of n-LDL in o-OLETF MCs was similar to that in LETO MCs. It has been shown that low concentrations of LDL induce proliferation of rat MCs but high concentrations (100 μg/mL) of it are instead cytotoxic [45], as we found in OLETF and LETO MCs. It is generally considered that LDL concentrations of 100 to 500 μg/mL are well within the physiological range in the plasma of non-diabetic rats [45]. In OLETF rats that develop hyperlipidemia after 8 weeks of age [6], the plasma lipoprotein levels are expected to be increased with age. It is unknown, however, what the prevailing concentration of LDL is for mesangial cells in vivo, because the passage of LDL particles into the mesangial area may be influenced by the accessibility to fenestrated endothelium and by charged endothelium [45].

Oxidized-LDL is produced by conversion from n-LDL by free radicals excreted by macrophages or intrinsic glomerular cells [33, 46–48]. Cultured MCs from non-diabetic rats have been demonstrated to bind and incorporate ox-LDL to a greater extent than n-LDL [41], which results in the alterations of eicosanoid synthesis and cytokine production, influencing local glomerular hemodynamics, vascular permeability and ECM production [41, 49]. LDL binding studies indicated that y-OLETF MCs possess a relatively low concentration of scavenger receptors compared with LDL receptors, which may be related to the poor response of DNA synthesis to ox-LDL. In contrast, o-OLETF MCs expressed high amounts of scavenger receptors and showed the down-regulation of LDL receptors. Following stimulation with n-LDL or ox-LDL, o-OLETF MCs produced ECM proteins, while y-OLETF synthesized rather small amounts of these proteins. It appears that functional changes of MCs with respect to the response to n-LDL/ox-LDL occur with the development of diabetes in OLETF rats. Although the precise mechanisms of the functional switch in OLETF MCs are unclear, the switch may be explained by a transition from cellular expression of LDL receptors to expression of scavenger receptors and also by co-expression of TGF-β in o-OLETF MCs, but not in y-OLETF MCs. Furthermore, it is speculated that the intracellular signaling processes are different in MCs from y-OLETF and o-OLETF rats. Stimulation by n-LDL or modified LDL has been reported to induce activation of extracellular signal-regulated kinases (ERK), one of the mitogen-activated protein kinase

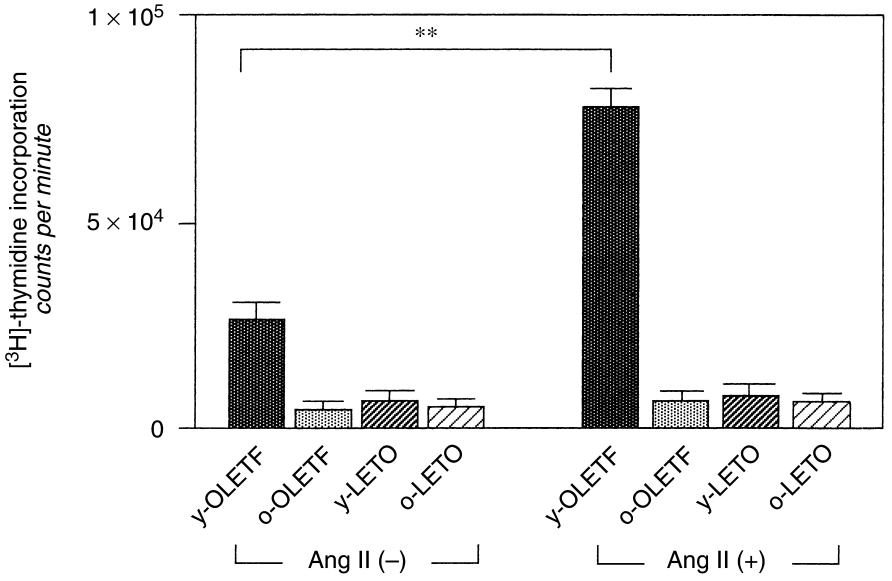

Fig. 9. DNA synthesis in OLETF and LETO MCs exposed to angiotensin II (Ang II 10^{-7} mol/L) for 18 hours. [3H]-thymidine incorporation was high in unstimulated y-OLETF MCs, and was further increased by exposure to Ang II, while it was low in o-LETF and LETO MCs with or without Ang II exposure. Symbols and bars represent means ± SE of 5 separate experiments ($N = 3$ rats per group); $^{**}P < 0.01$.

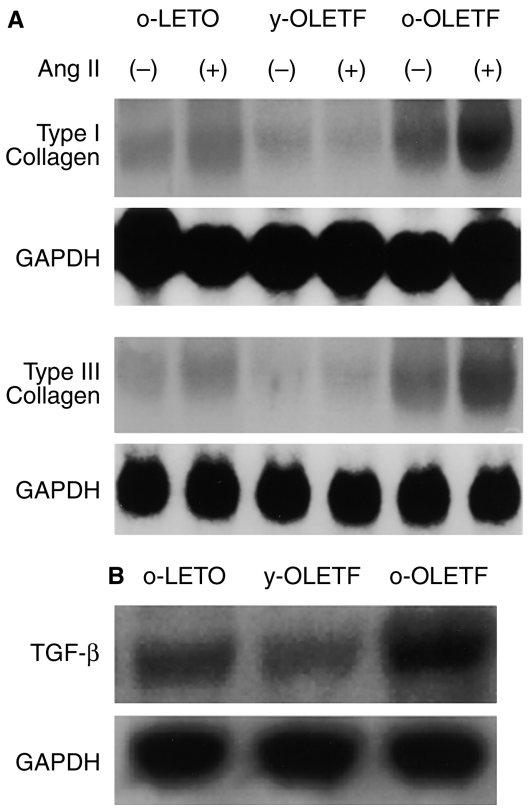

Fig. 10. Northern blot analysis for type I and III collagen mRNA (*A*) and TGF-β mRNA (*B*) in Ang II-stimulated MCs from OLETF and LETO rats. (A) Incubation with Ang II (Ang II+) resulted in up-regulation of type I and III collagen mRNAs in o-OLETF MCs, but not in y-OLETF and LETO MCs. (B) After incubation of MCs with Ang II, TGF-β mRNA expression was stronger in o-OLETF MCs than in y-OLETF or o-LETO MCs. In untreated MC cultures in FCS, TGF-β mRNA expression was minimal in OLETF and LETO rats (as shown in Fig. 8B).

signaling pathways, in non-diabetic rat MCs [50], which affects cell proliferation and also matrix production. The activation of this pathway may contribute to MC proliferation or ECM synthesis in diabetic conditions as well. Gene induction downstream of the ERK cascade might be different in y-OLETF and o-OLETF rats, thus inducing different responses to the same stimulus.

Various lines of evidence indicate that the renin-angiotensin system is locally activated in diabetic nephropathy, thus influencing parenchymal cell functions and hemodynamics in the kidney [51, 52]. In several human studies, angiotensin-converting-enzyme inhibitors or Ang II receptor blockades have been demonstrated to be renoprotective, independent of their systemic antihypertensive actions [53, 54]. Ang II contributes to control of hypertrophy, DNA synthesis and ECM production in glomerular cells [9, 52, 55]. These actions are exerted in part by synergy with other growth factors such as TGF-β or by co-inhibition of proteolytic enzymes to retard the degradation of ECM [9, 56]. In this study, y-OLETF MCs stimulated with Ang II showed a significant increase in DNA synthesis compared with o-OLETF and LETO MCs. In contrast, o-OLETF MCs stimulated with Ang II produced type I and type III collagen and showed the enhancement of TGF-β expression, whereas ECM synthesis was not markedly affected by Ang II in y-OLETO and LETO MCs. The precise mechanisms of the shift from cell proliferation toward ECM production in Ang II-stimulated MCs are unclear. In the late stage of diabetes in OLETF rats, prolonged hyperglycemia or other metabolic abnormalities might facilitate expression of other relevant growth factors such as TGF-β. Similar to our findings, Yagi et al reported that, in OLETF rats, glomerular type I collagen mRNA and TGF-β mRNA

were up-regulated at diabetic stage (30- and 54-weeks-old), but not at the prediabetic stage (14-weeks-old) [57]. Previous reports [19, 27, 30–32, 57] and our present findings collectively indicate that stage-specific gene regulation is induced in multipotential MCs of diabetic rats. The precise mechanisms of this process has not been fully elucidated, but expression of specific receptors, as observed in n-LDL/ox-LDL, coexpression of TGF-β, as we found in o-OLETF MCs stimulated with ox-LDL and Ang II, and other mechanisms such as different activation or control of genes downstream of intracellular signals in y-OLETF and o-OLETF rats may be involved.

In summary, our observations showed that, upon stimulation with n-LDL/ox-LDL and Ang II, functional transformation from proliferation to ECM biosynthesis occurred in cultured OLETF MCs during the evolution of diabetes. The status of these elements in diabetic conditions in vivo remain speculative, but in vitro experiments using cultured MCs may reflect certain processes of abnormal regulation of proliferation and ECM production in vivo. Further studies are needed to demonstrate that intervention with agents modifying MC responses in the early phase can retard or prevent the progression of diabetic nephropathy.

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REFERENCES

1. Rate R, Knowler C, Morse H, *et al*: Diabetes mellitus in Hopi and Navajo Indians. *Diabetes* 32:894–899, 1983
2. Selby JV, FitzSimmons SC, Newman JM, *et al*: The natural history and epidemiology of diabetic nephropathy. Implications for prevention and control. *JAMA* 263:1954–1960, 1990
3. Mauer SM, Steffes MW, Ellis EN, *et al*: Structural-functional relationships in diabetic nephropathy. *J Clin Invest* 74:1143–1155, 1984
4. Mauer S, Steffes M, Brown D: Animal models of diabetic nephropathy. *Adv Nephrol* 18:23–42, 1979
5. Kawano K, Hirashima T, Mori S, *et al*: A new rat strain with non-insulin dependent diabetes mellitus, "OLETF" rat. *News Lett* 25:24–26, 1991
6. Kawano K, Hirashima T, Mori S, *et al*: Spontaneous long-term hyperglycemic rat with diabetic complications; Otsuka Long-Evans Tokushima Fatty (OLETF) strain. *Diabetes* 41:1422–1428, 1992
7. Flyvberg A, Gronbaek H, Bak M, *et al*: Diabetic kidney disease: The role of growth factors. *Nephrol Dial Transplant* 13: 1104–1107, 1998
8. Kikkawa R, Haneda M: Pathogenesis of diabetic nephropathy. *Clin Exp Nephrol* 1:3–11, 1997
9. Wolf G, Ziyadeh F: Molecular mechanisms of diabetic renal hypertrophy. *Kidney Int* 56:393–405, 1999
10. Harper P, Robinson J, Hoover R, *et al*: Improved methods for culturing rat glomerular cells. *Kidney Int* 26:875–880, 1984
11. Miyazato H, Takemura T, Hino S, *et al*: Glucocorticoid-induced apoptosis of rat mesangial cells in culture. *Clin Exp Nephrol* 2:12–17, 1998
12. Takemura T, Murata Y, Hino S, *et al*: Heparin-binding EGF-like growth factor is expressed by mesangial cells and involved in mesangial proliferation in glomerulonephritis. *J Pathol* 189:431–438, 1999
13. Takemura T, Kondo S, Homma T, *et al*: The membrane-bound form of heparin-binding epidermal growth factor-like growth factor promotes survival of cultured renal epithelial cells. *J Biol Chem* 272:31036–31042, 1997
14. Akagi Y, Isaka Y, Arai M, *et al*: Inhibition of TGF−β expression by antisense oligonucleotides suppressed extracellular matrix accumulation in experimental glomerulonephritis. *Kideny Int* 50:148–155, 1996
15. Goldstein J, Basu S, Brown M: Receptor-mediated endocytosis of low-density lipoprotein in cultured cells. *Methods Enzymol* 98: 241–260, 1983
16. Wasserman J, Santiago A, Rifici V, *et al*: Interactions of low density lipoprotein with rat mesangial cells. *Kidney Int* 35:1168–1174, 1989
17. Floege J, Burns M, Alpers C, *et al*: Glomerular cell proliferation and PDGF expression precede glomerular sclerosis in the remnant kidney model. *Kidney Int* 41:297–309, 1992
18. Takemura T, Okada M, Akano N, *et al*: Proto-oncogene expression in human glomerular diseases. *J Pathol* 178:343–351, 1996
19. Young B, Johnson R, Alpers C, *et al*: Cellular events in the evolution of experimental diabetic nephropathy. *Kidney Int* 47: 935–944, 1995
20. Yoshioka K, Takemura T, Murakami K, *et al*: Transforming growth factor-β protein and mRNA in glomeruli in normal and diseased human kidneys. *Lab Invest* 68:154–163, 1993
21. Takemura T, Yoshioka K, Aya N, *et al*: Apolipoproteins and lipoprotein receptors in glomeruli in human kidney diseases. *Kidney Int* 43:918–927, 1993
22. Yoshioka K, Takemura T, Murakami K, *et al*: In situ expression of cytokines in IgA nephritis. *Kideny Int* 44:825–833, 1993
23. Osterby R: Morphological studies of the peripheral glomerular basement membrane in early juvenile diabetes. I. Development of initial basement membrane thickening. *Diabetologia* 8: 84–92, 1972
24. Osterby R, Gundersen H: Glomerular size and structure in diabetc mellitus. I. Early abnormalities. *Diabetologia* 11:225–229, 1975
25. Seyer-Hansen K: Renal hypertrophy in streptozotocin-diabetic rats. *Clin Sci Mol Med* 51:551–555, 1976
26. Romen W, Takahashi A: Autoradiographic studies on the proliferation of glomerular and tubular cells of rat kidney in early diabetes. *Virchows Arch B* 40:339–345, 1982
27. Mahadevan P, Larkins R, Fraser J, Dunlop M: Effect of prostaglandin E2 and hyaluronan on mesangial cell proliferation: A potential contribution to glomerular hypercellularity in diabetes. *Diabetes* 45:44–50, 1996
28. Baserga R: Growth regulation by the PCNA gene. *J Cell Sci* 98: 433–436, 1991
29. Kaczmarek L: Protooncogene expression during the cell cycle. *Lab Invest* 54:365–376, 1986
30. Nakamura T, Fukui M, Ebihara I, *et al*: mRNA expression of growth factors in glomeruli from diabetic rats. *Diabetes* 42:450–456, 1993
31. Shankland S, Scholey J, Ly H, Thai K: Expression of growth-related protooncogens during diabetic renal hypertrophy. *Kidney Int* 47:782–788, 1995
32. Wolf G, Sharma K, Chen Y, *et al*: High glucose-induced proliferation in mesangial cell is reversed by autocrine TGF-β. *Kidney Int* 42:647–656, 1992
33. Moorhead J, Wheeler D, Varghese Z: Glomerular structures and lipids in progressive renal disease. *Am J Med* 87(5N):5-25N–25-29N, 1989
34. Schmitz P, Kasiske B, O'Donnell M, Keane W: Lipids and progressive renal injury. *Semin Nephrol* 9:354–369, 1989
35. Kasiske B, Cleary M, O'Donnell M, Keane W: Mechanisms of glomerular injury in the obese Zucker rat. *Kidney Int* 33:667–672, 1988
36. Diamond J, Karnovsky M: Exacerbation of chronic aminonucleo-

side nephrosis by dietary cholesterol supplementation. *Kidney Int* 32:671–678, 1987
37. Wilson P, Kannel W, Anderson K: Lipids, glucose intolerance, and vascular disease: The Framingham study. *Monogr Atheroscler* 13:1–11, 1985
38. Ha H, Kamanna V, Kirchenbaum M, Kim K: Role of glycated low density lipoprotein in mesangial extracellular matrix synthesis. *Kidney Int* 54(Suppl):S54–S59, 1997
39. Lyons T, Li W, Wells-Knecht M, Jokl R: Toxicity of mildly modified low-density lipoproteins to cultured retinal capillary endothelial cells and pericytes. *Diabetes* 43:1090–1095, 1994
40. Grone H-J, Walli A, Grone E, *et al*: Receptor mediated uptake of apo B and apo E rich lipoproteins by human glomerular epithelial cells. *Kidney Int* 37:1449–1459, 1990
41. Coritsidis G, Rifici V, Gupta S, *et al*: Preferential binding of oxidized LDL to rat glomeruli in vivo and cultured mesangial cells in vitro. *Kidney Int* 39:858–866, 1991
42. Steinberg D, Parthasara S, Carew T, *et al*: Beyond cholesterol: Modification of low-density lipoprotein that increase its atherogenecity. *N Engl J Med* 320:915–924, 1989
43. Iverius P-H: The interaction between human plasma lipoproteins and connective tissue gycosaminoglycans. *J Biol Chem* 247:2607–2613, 1972
44. Michael A, Keane W, Raji L, *et al*: The glomerular mesangium. *Kidney Int* 17:141–154, 1980
45. Wheeler D, Persaud J, Fernando R, *et al*: Effects of low-density lipoproteins on mesangial cell growth and viability in vitro. *Nephrol Dial Transplant* 5:185–191, 1990
46. Brown M, Goldstein J: Lipoprotein metabolism in the macrophage: Implications for cholesterol deposition in atherosclerosis. *Annu Rev Biochem* 52:223–261, 1983
47. Chen H, Tan M, Guh J, *et al*: Native and oxidized low-density lipoproteins enhance superoxide production from diabetic rat glomeruli. *Kidney Blood Press Res* 23:133–137, 2000
48. Ishiyama A, Atarashi K, Minami M, *et al*: Role of free radicals in the pathogenesis of lipid-induced glomerulosclerosis in rats. *Kidney Int* 55:1348–1358, 1999
49. Striker G, Lange M, Mckay K, *et al*: Glomerular cells in vitro. *Adv Nephrol* 16:169–186, 1987
50. Jenkins A, Velarde V, LKlein R, *et al*: Native and modified LDL activate extracellular signal-regulated kinases in mesangial cells. *Diabetes* 49:2160–2169, 2000
51. Anderson S: Role of local and systemic angiotensin in diabetic renal disease. *Kidney Int* 63(Suppl):S107–S110, 1997
52. Ichikawa I, Harris R: Angiotensin actions in the kidney: Renewed insight into the old hormone. *Kidney Int* 40:583–596, 1991
53. Anderson S, Tarnow L, Rossing P, *et al*: Renoprotective effects of angiotensin II receptor blockade in type 1 diabetic patients with diabetic nephropathy. *Kidney Int* 57:601–606, 2000
54. Lewis E, Hunsicker L, Bain R, *et al*: The effect of angiotensin-converting-enzyme inhibition on diabetic nephropathy. *N Engl J Med* 329:1456–1462, 1993
55. Wolf G, Haberstroh U, Neilson E: Angiotensin II stimulate the proliferation and biosynthesis of type I collagen in cultured murine mesangial cells. *Am J Pathol* 140:95–107, 1992
56. Kagami S, Border W, Miller D, Noble N: Angiotensin II stimulates extracellular matrix protein synthesis through induction of transforming growth factor-β expression in rat glomerular mesangial cells. *J Clin Invest* 93:2431–2437, 1994
57. Yagi K, Kim S, Wanibuchi H, *et al*: Characteristics of diabetes, blood pressure, and cardiac and renal complications in Otsuka Long-Evans Tokushima fatty rats. *Hypertension* 29:728–735, 1997